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Effect of vitamin C supplements on antioxidant defence and stress proteins in human lymphocytes and skeletal muscle

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Oxidative stress induces adaptations in the expression of protective enzymes and heat shock proteins (HSPs) in a variety of tissues. We have examined the possibility that supplementation of subjects with the nutritional antioxidant, vitamin C, influences the ability of lymphocytes to express protective enzymes and HSPs following exposure to an exogenous oxidant and the response of skeletal muscle to the physiological oxidative stress that occurs during exercise *in vivo*. Our hypothesis was that an elevation of tissue vitamin C content would reduce oxidant-induced expression of protective enzymes and HSP content. Lymphocytes from non-supplemented subjects responded to hydrogen peroxide with increased activity of superoxide dismutase (SOD) and catalase, and HSP60 and HSP70 content over 48 h. Vitamin C supplementation at a dose of 500 mg day^{-1} for 8 weeks was found to increase the serum vitamin C concentration by ~50 %. Lymphocytes from vitamin C-supplemented subjects had increased baseline SOD and catalase activities and an elevated HSP60 content. The SOD and catalase activities and the HSP60 and HSP70 content of lymphocytes from supplemented subjects did not increase significantly in response to hydrogen peroxide. In non-supplemented subjects, a single period of cycle ergometry was found to significantly increase the HSP70 content of the vastus lateralis. Following vitamin C supplementation, the HSP70 content of the muscle was increased at baseline with no further increase following exercise. We conclude that, in vitamin C-supplemented subjects, adaptive responses to oxidants are attenuated, but that this may reflect an increased baseline expression of potential protective systems against oxidative stress (SOD, catalase and HSPs).

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Reactive oxygen species (ROS) are generated as part of normal metabolism in mammalian cells. The majority of cellular ROS appear to be generated by mitochondria, but in phagocytic cells NAD(P)H oxidase systems additionally generate substantial amounts of ROS during activation (Halliwell & Gutteridge, 1989). Skeletal muscle generates significant amounts of oxidants during aerobic contractile activity. Exercise can increase oxygen utilisation 200-fold above resting levels in active muscle fibres (Keul *et al.* 1972) and it has been suggested that superoxide production increases with this large increase in oxygen flux through muscle mitochondria during exercise (Davies *et al.* 1982). This is supported by data from our laboratory and others indicating that skeletal muscle cells release superoxide (McArdle *et al.* 2001) and generate hydroxyl radicals (O'Neill *et al.* 1996) in the extracellular fluid during contraction. Skeletal muscle also contains nitric oxide (NO) synthases and releases NO to the extracellular fluid during contractile activity (Balon & Nadler, 1994).

Evidence from both animal and human studies indicates that many cell types adapt to increased exposure to oxidants to reduce the risk of damage to the tissue (Niwa *et al.* 1993; Marini *et al.* 1996; Jones *et al.* 1999; McArdle *et al.* 2001). Lymphocytes increase their activity of SOD, catalase (CAT) and glutathione peroxidase in response to endogenous oxidants (Barnett *et al.* 1995) and an acute bout of exercise increases the activities of SOD, glutathione peroxidase, glutathione reductase and catalase in skeletal muscle of rats (Ji, 1993). Longer-term exercise training also appears to increase the activity of several antioxidant enzymes, such as SOD and CAT (Higuchi *et al.* 1985) or glutathione peroxidase (Ji, 1993) in muscle, although these are not consistent findings (Alessio & Goldfarb, 1988). In humans, exercise training has been reported to increase skeletal muscle SOD activities (Jenkins *et al.* 1984) and the activities of various protective enzymes in blood (Robertson *et al.* 1991).

In addition to adaptive changes in protective enzymes, oxidative and other stresses to cells are known to induce increased production of stress or heat shock proteins (HSPs). These proteins are an important component of the cellular protective response. This occurs in blood cells such as lymphocytes (Marini *et al.* 1996), and recent data also indicate that an increase in muscle HSP content

occurs following exercise in rats (Salo *et al.* 1991), mice (McArdle *et al.* 2001) and humans (Khassaf *et al.* 2001). HSPs act as molecular chaperones facilitating the correct folding of newly synthesised cellular proteins and translocation to cellular compartments (Fiege *et al.* 1996). Studies in a variety of tissues indicate that prior stimulation of the synthesis of heat shock proteins protects tissues against a variety of (normally damaging) stresses, such as ischaemic–reperfusion injury or intracellular calcium overload (Marber *et al.* 1995).

An increase in oral intake of vitamin C has been proposed to be potentially beneficial in reducing oxidative damage to tissues by chemical reduction of oxidant species (for recent references see Wardle, 1999; Simon *et al.* 2001; Thompson *et al.* 2001). The increasing recognition that oxidative stress induces the increased expression of protective enzymes and HSPs in tissues has prompted us to examine the relationship between vitamin C supplementation and the ability of lymphocytes to express protective enzymes and HSPs in response to exogenous oxidants. In addition, in the same subjects, we have examined the effect of vitamin C supplements on the responses of skeletal muscle to the physiological oxidative stress during exercise *in vivo*. Our hypothesis was that an elevation of tissue vitamin C content would reduce the oxidant-induced increase in expression of protective enzymes and HSPs in lymphocytes *ex vivo* and in skeletal muscle following exercise *in vivo*.

METHODS

Subjects

Sixteen healthy, untrained male volunteers participated in the study. All were non-smokers and not taking any routine medication or vitamin supplements and none participated in regular sport or exercise training. Their heights, masses and ages were 1.79 ± 0.03 m, 81.6 ± 4.2 kg and 28 ± 2 years (means ± S.E.M.), respectively. The protocol was approved by the Liverpool Research Ethics Committee and all subjects gave informed written consent. The study was in accordance with the Declaration of Helsinki.

Subjects were randomised to receive either 0.5 g day^{-1} vitamin C (Roche, Basle, Switzerland, > 99 % ascorbic acid) or no supplements for 8 weeks. Two days prior to beginning the supplement, subjects gave a blood sample and a muscle biopsy sample, and undertook an exercise protocol followed by a second muscle biopsy 2 days later. Eight weeks later the blood and muscle sampling and the exercise test were repeated.

Preparation of lymphocytes

For preparation of lymphocytes, heparinised blood samples were obtained from both unsupplemented and supplemented subjects prior to exercise. Blood was centrifuged (1500 *g*) and plasma removed. The cell pellet was reconstituted to the original volume with RPMI 1640 medium (Invitrogen Ltd, Paisley, UK) and approximately 8 ml was layered onto 3 ml Ficol plaque (Sigma, Poole, UK) and centrifuged at 1000 *g* for 30 min at room temperature. The lymphocyte band was harvested, resuspended in 12 ml RPMI and centrifuged at 1000 *g* for 15 min. The lymphocyte pellet was again harvested and washed in RPMI. Cells were cultured overnight in RPMI and suspended cells were re-plated. Preliminary studies indicated that there was no significant change in the vitamin C content of lymphocytes during this procedure. The purified lymphocytes were maintained in RPMI, 10 % fetal calf serum and glutamine and treated with either 15 or 25 μM H_2O_2 for 30 min. Aliquots of cells were taken 12, 24 and 48 h later and analysed for SOD and CAT activities, and HSP60 and HSP70 content. Viability of cells was assessed by trypan blue exclusion.

Exercise protocol

Each subject exercised at a cadence of 70 r.p.m., using a single leg, on a friction loaded cycle ergometer (Monark 864, Sweden) which was modified specifically for this purpose (Khassaf *et al.* 2001). Prior to randomisation, an incremental work test was performed to determine peak oxygen uptake, during which the workload was increased by 35 W every 4 min to volitional exhaustion. Expired air was monitored continuously during exercise using an on-line gas analysis system (Vmax 20 cardio-pulmonary exercise testing instrument, SensorMedics Corp., Yorba Linda, CA, USA). Average oxygen uptake was determined over the last 45 s of each workload and in the 45 s prior to test termination. As the subjects were unaccustomed to this exercise protocol, a second incremental work test was performed within a week of the first test to assess the reliability of the measurements. These were used to calculate the workload to elicit an oxygen uptake of 70 % peak oxygen uptake ($\dot{V}_{O_2,peak}$).

During the experimental protocols the subjects each cycled at 70 % $\dot{V}_{O_2,peak}$ for 45 min using the leg contralateral to that used for the preliminary incremental tests. Pre-exercise, resting oxygen uptake was determined from respiratory data collected during the 10 min prior to exercise and oxygen uptake was measured over the last minute of each 5 min period during exercise. Heart rate was recorded prior to and throughout the exercise test.

Blood samples and muscle biopsies from vastus lateralis muscle of the exercised leg were taken 2 days prior to the exercise protocol. Two days following the exercise, further muscle biopsy samples were obtained. Biopsy samples were taken under local anaesthesia (2 % lignocaine), using a Bergstrom-type needle (6.5 mm diameter). Samples (approximately 100 mg) were obtained and immediately frozen in liquid nitrogen and stored at −70 °C until analysed.

Biochemical methods

Lymphocytes were disrupted by sonication in saline and dispensed into aliquots for the different analyses. Frozen muscle biopsy samples were ground under liquid nitrogen and the powdered sample was dispensed into aliquots. For analysis of HSPs, powdered muscle was homogenised in a range of protease inhibitors and the lymphocyte lysate was diluted in the same inhibitors. Samples were then centrifuged (10 000 *g*) at 4 °C and the supernatant analysed for total protein content using the bicinchoninic acid method (Sigma). A quantity (50 μg) of total protein was separated by SDS-PAGE followed by Western blotting. CAT activity was measured by following the kinetic decomposition of H_2O_2 at 240 nm, using a method derived from Claiborne (1985). Total SOD activity was measured using the method of Crapo *et al.* (1984). The content of HSP60 and HSP70 was analysed using monoclonal antibodies obtained from Bioquote Ltd (Yorkshire, UK) and Amersham Biosciences UK Ltd (Khassaf *et al.* 2001). Bands were visualised on X-ray film using the ECL chemiluminescent detection system (Amersham). Membranes were exposed to film for three to four different

exposure times to ensure that saturation of film had not occurred. Samples from each subject were applied to the same gel and the intensity of staining for individual HSPs was quantified by densitometry (Analytical Imaging Station, Imaging Research Inc., Ontario, Canada). The HSP content was expressed as a percentage of the pre-exercise content for each subject (Khassaf *et al.* 2001).

Statistical analyses

Inter-individual variability in all measures was high and data are therefore presented as means ± S.E.M. of the percentage of the pre-exercise value from the non-supplemented data. Initial comparisons between data sets were undertaken by analysis of variance, followed by comparisons between means using the Bonferroni correction.

RESULTS

Subjects tolerated the vitamin C supplementation well with no reports of problems with compliance. This dose (500 mg day^{-1}) and duration of vitamin C supplementation were chosen since in previous studies we have shown that it leads to an increase in tissue vitamin C content (F. McArdle *et al.* 2002). In the current study, 8 weeks of supplementation with vitamin C at a dose of 500 mg day^{-1} was found to increase the mean serum vitamin C concentration from 3.8 ± 0.3 μg ml^{-1} at baseline to 5.2 ± 0.5 μg ml^{-1} in comparison with pre-supplementation values. These values are similar to those previously reported by other investigators using this supplementary dose of vitamin C (Cooke *et al.* 1998). Neither serum vitamin C concentrations nor other measures varied significantly in the placebo group and hence data are not presented in detail. Peak oxygen uptake did not vary between the first and second preliminary incremental work tests in either placebo or test groups in all subjects.

Baseline lymphocyte activities of CAT and SOD were variable between non-supplemented subjects with values of 189 ± 53 and 5.4 ± 2.0 U (mg protein)$^{-1}$, respectively. Lymphocytes from non-supplemented subjects were

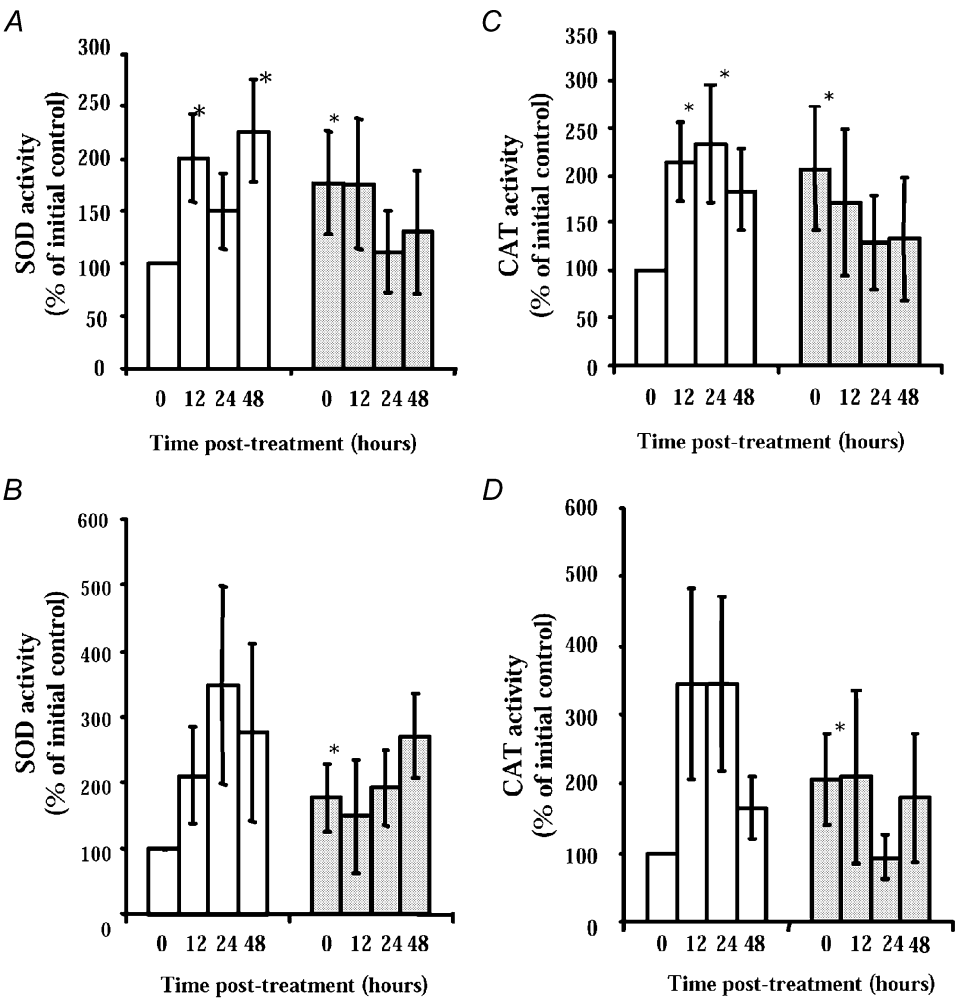

Figure 1

Lymphocyte superoxide dismutase (SOD) activity following exposure to 15 μM H2O2 (*A*) and 25 μM H_2O_2 (*B*), and catalase activity following exposure to 15 μM H_2O_2 (*C*) and 25 μM H_2O_2 (*D*) in subjects prior to (open bars), and following 8 weeks of supplementation with 500 mg day^{-1} vitamin C (grey bars). Data are presented as means ± S.E.M. * Values significantly different from pre-stimulation, non-supplemented values.

found to respond to either 15 or 25 μM H_2O_2 by increasing SOD and CAT activities, and HSP60 and HSP70 content over the following 48 h (Figs 1–3). Treatment with 15 μM H_2O_2 induced significant changes in all four responses, but 25 μM induced more variable responses. Preliminary experiments indicated that these doses of H_2O_2 did not cause loss of cell viability in the lymphocytes and that the SOD and CAT activities, or HSP60 and HSP70 content, did not change in unstressed lymphocytes over 48 h in culture (data not shown). Vitamin C supplementation had a variable effect on the responses of lymphocytes to H_2O_2.

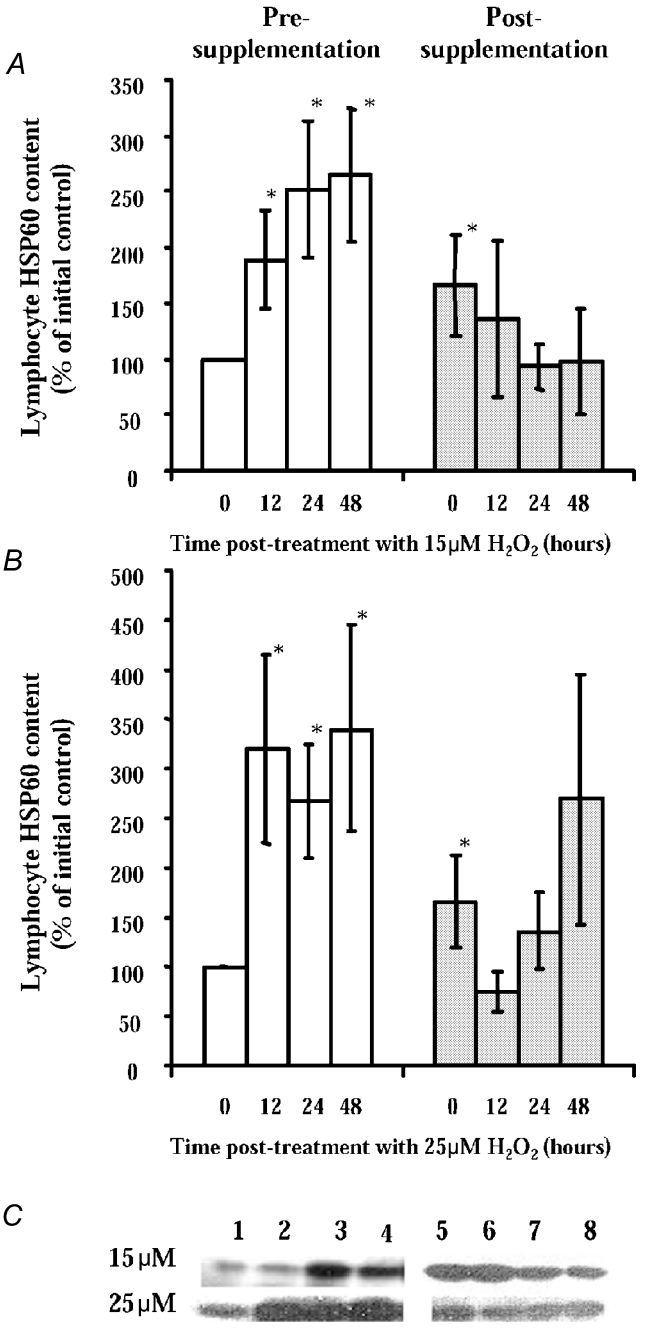

Figure 2

Lymphocyte HSP60 content following exposure to 15 μM (*A*) and 25 μM H_2O_2 (*B*) in volunteer subjects prior to (open bars), and after 8 weeks supplementation with 500 mg day^{-1} vitamin C (grey bars). Data are presented as means ± S.E.M. * Values significantly different from pre-stimulation, non-supplemented values. *C*, example Western blots are also shown for pre-supplementation (lanes 1–4) and post-supplementation (lanes 5–8) samples.

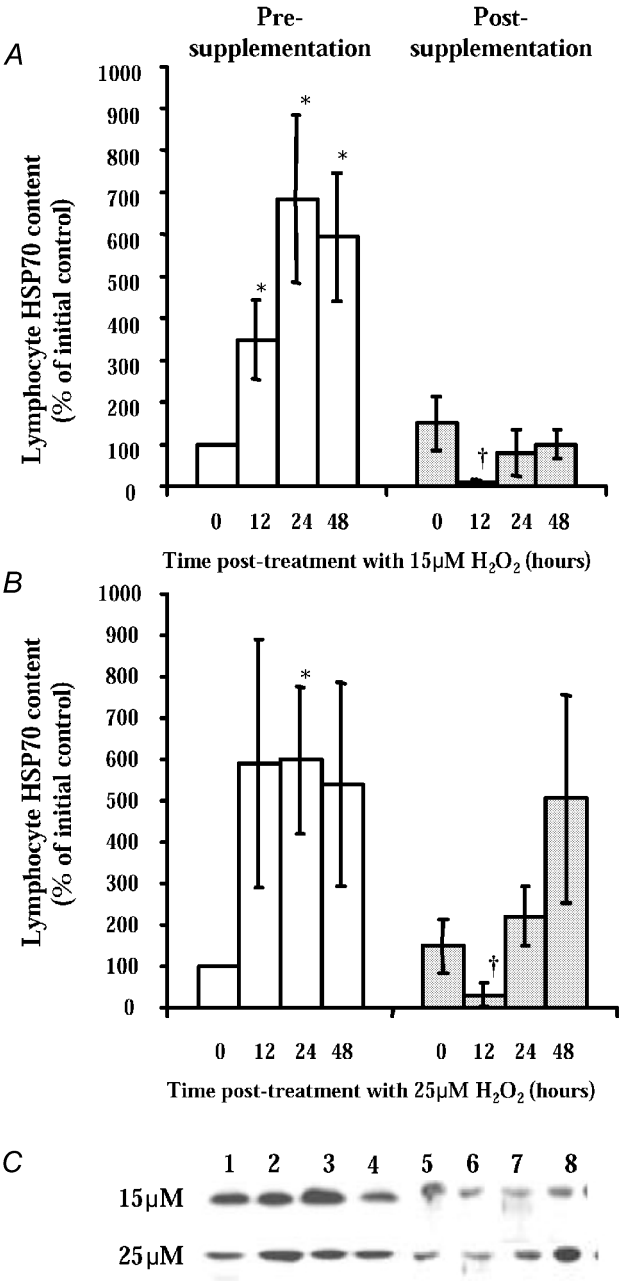

Figure 3

Lymphocyte HSP70 content following exposure to 15 μM (*A*) and 25 μM H_2O_2 (*B*) in volunteer subjects prior to (open bars), and after 8 weeks supplementation with 500 mg day^{-1} vitamin C (grey bars). Data are presented as means ± S.E.M. * Values significantly different from pre-stimulation, non-supplemented values; † value significantly different from data from non-supplemented subjects at the same time post-H_2O_2. *C*, example Western blots are also shown for pre-supplementation (lanes 1–4) and post-supplementation (lanes 5–8) samples.

Baseline activities of both SOD and CAT were elevated in the vitamin C supplemented group and there were no further significant increases on stimulation with either 15 or 25 μM H_2O_2 (Fig. 1).

Similar patterns were seen for HSP60 and HSP70 expression. Baseline content of HSP60 was significantly higher in the vitamin C-supplemented subjects and they also showed a dramatic attenuation in H_2O_2-induced expression of both HSPs (Figs 2 and 3). The effect was particularly marked for the HSP70 content of lymphocytes treated with H_2O_2 (Fig. 3) where the HSP70 content of lymphocytes from vitamin C-supplemented subjects was greatly reduced in comparison with values from the non-supplemented period at 12 h following the H_2O_2 stress.

The period of exercise induced a significant rise in the HSP70 content of the exercised vastus lateralis muscle at 2 days post-exercise (Fig. 4). Muscle HSP60 content showed a tendency to increase, although this was not statistically significant. Following vitamin C supplementation, the pre-exercise content of HSP70 was significantly increased with no further increase following exercise (Fig. 4*A*). No significant change in muscle HSP60 content was seen following vitamin C supplements. No changes in muscle SOD or CAT activities were seen at 2 days before or after exercise in either the placebo or vitamin C-treated groups (results not shown in detail).

DISCUSSION

All nucleated cells respond to stress by upregulation of a complex array of defence mechanisms and the data presented here have indicated that vitamin C supplementation causes an increase in the baseline expression of these defences and modifies the responses of both muscle cells and lymphocytes to oxidative stress.

The data confirm that lymphocytes respond to low levels of exogenous oxidative stress by upregulation of the protective enzymes SOD and CAT and of HSPs in agreement with previous studies (Niwa *et al.* 1993; Barnett *et al.* 1995). These proteins are able to protect the cells against potential damage from subsequent oxidative (Barnett *et al.* 1995) and other stresses (Samali *et al.* 2001). The responses to the higher (25 μM) concentration of H_2O_2 were more variable than to the lower dose, although most cultures clearly responded at both concentrations. The isoform(s) of SOD that were induced by the oxidative stress were not determined because of lack of sample, but by analogy with previous data it seems probable that the major isoform induced was MnSOD (Bravard *et al.* 1999; Pajovic *et al.* 2000; Joksic *et al.* 2000).

The overall effect of vitamin C supplementation appeared to be to increase the baseline lymphocyte SOD and CAT activities and HSP60 content, and to attenuate the increase in expression of these substances that normally follows exposure to oxidants. The attenuation was particularly marked for HSP70 where levels following H_2O_2 exposure were much lower following vitamin C supplementation.

The effects of vitamin C on the HSP responses of skeletal muscle to exercise differed depending upon the specific

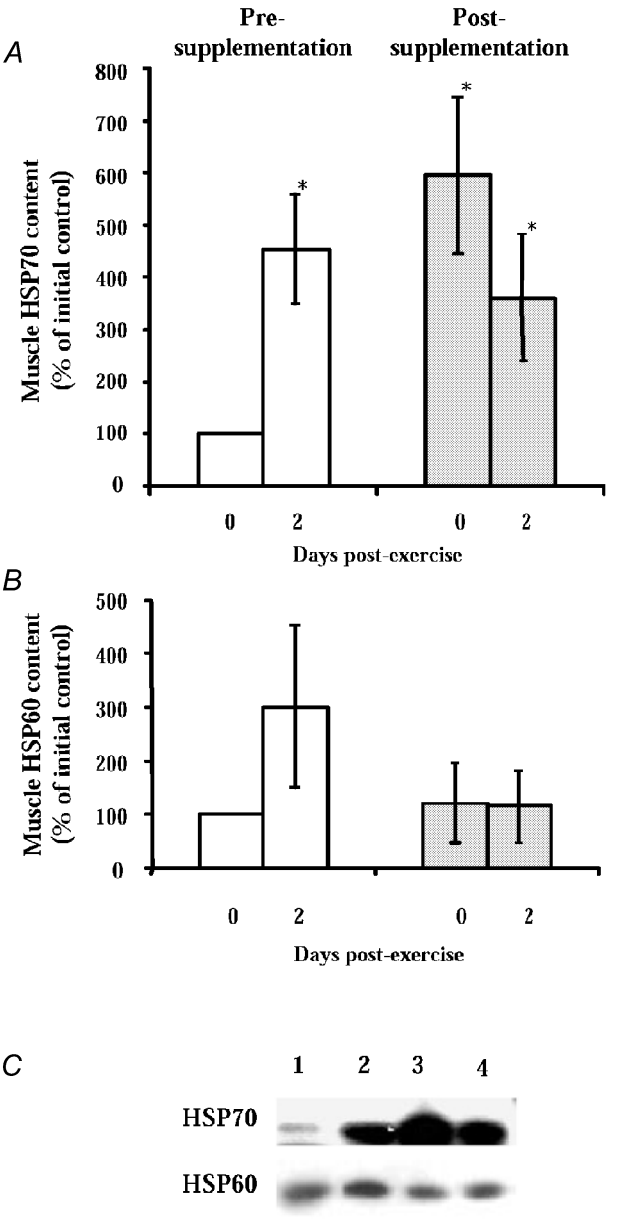

Figure 4

Relative contents of HSP70 (*A*) and HSP60 (*B*) in the vastus lateralis muscles of a group of volunteer subjects prior to, and at 2 days following, 45 min of exercise on a cycle ergometer. The effect of supplementation for 8 weeks with 500 mg day^{-1} vitamin C is also shown (grey bars). Data are presented as means ± S.E.M. * Values significantly different from pre-stimulation, non-supplemented values. *C*, example Western blots are shown for pre-supplementation (lanes 1 and 2) and post-supplementation samples (lanes 3 and 4).

HSP. For HSP70, the supplementation induced a significant elevation in the pre-exercise HSP70 content and the post-exercise increase in muscle content was abolished by vitamin C supplements. HSP60 showed no significant rise following exercise, in contrast to previous data (Khassaf *et al.* 2001).

A great deal of epidemiological and some experimental data indicate that high intakes of vitamin C can provide protection against oxidative damage *in vivo* although whether this occurs in all situations has been the subject of considerable discussion (see Halliwell & Gutteridge, 1989). The data reported here suggest that the dose of vitamin C supplement used may induce additional defences against oxidative damage, through an increase in lymphocyte SOD and CAT activities, HSP60 content and muscle HSP70 content. Previous studies have reported that the dose of vitamin C supplement used here increased tissue vitamin C content, but had variable effects on markers of oxidation depending upon on the marker and tissue examined. Thus in our previous work, 500 mg day^{-1} vitamin C appeared to lead to either a decrease or an increase in markers of oxidation in a single tissue (the skin) depending upon whether the malonaldehyde or the glutathione/protein thiol contents were used as the marker of oxidation (F. McArdle *et al.* 2002). At a similar level of vitamin C supplementation, we also found no effect on plasma malonaldehyde content, before or after exercise (Thompson *et al.* 2001). Published data from other laboratories also show the same variability in response depending upon the marker and tissue in which they were measured (e.g. see Wen *et al.* 1997; Cooke *et al.* 1998; Aghdassi *et al.* 1999; Carty *et al.* 2000).

The mechanisms are unclear by which vitamin C supplementation stimulated an increase in the baseline HSP70 content of unstressed muscle, the lymphocyte content of HSP60 and the activities of SOD and CAT. It has been proposed that vitamin C may exert pro-oxidant effects in some situations, acting to reduce ferric ions with the resulting ferrous species capable of catalysing formation of hydroxyl radical species from H_2O_2 (Halliwell & Gutteridge, 1989). Whether this process occurs *in vivo* is the subject of considerable debate. Podmore and colleagues (1998) reported that a similar level of vitamin C supplementation to that used here induced an increase in the 8-oxo, adenine content of lymphocyte DNA indicating an increased oxidation of this molecule, but these data have subsequently become the subject of considerable controversy (Poulsen *et al.* 1998; Levine *et al.* 1998). Analyses of other measures of oxidative damage in tissues of supplemented subjects do not provide clear data either in support or against this hypothesis (e.g. see F. McArdle *et al.* 2002; Thompson *et al.* 2001), but a pro-oxidant effect could potentially lead to an oxidation in tissues of the supplemented subjects, with subsequent adaptive increases in SOD and CAT activities and increase in HSP expression. Alternatively there is now evidence that supplementation with vitamin C and other antioxidants can directly induce modifications in gene expression with increased transcription of certain proteins (Catani *et al.* 2001). Such proteins may require increased expression of chaperones for correct cellular location and/or function, providing an alternative stimulus for the increased HSP expression.

In addition to the increase in baseline defences against oxidative damage, the vitamin C supplementation clearly attenuated the adaptive responses of lymphocytes to oxidative stress. Various mechanisms may have been involved. The vitamin C may have directly reduced the oxidant stress from the H_2O_2, potentially by reducing the free radical species generated from the H_2O_2. In addition the transcription factors involved in stimulation of SOD and CAT transcription (AP-1 and NFκB) and HSP transcription (HSF-1) are sensitive to the 'redox' state of the cell. Vitamin C may therefore act to maintain the reducing environment of the cell preventing activation of these transcription factors by H_2O_2 or derivatives (Jackson *et al.* 2002). The increased baseline activities of SOD and CAT and the HSP60 content of lymphocytes may also play a role in reducing lymphocyte responses to the exogenous oxidant. An increase in CAT activity would be predicted to decrease the intracellular content of H_2O_2 and an increase in HSP60 would potentially protect mitochondrial proteins against oxidative damage, changes that may reduce the stimulus to mount an adaptive response. We have previously speculated that increased generation of oxidants during exercise stimulates transcription of HSPs in skeletal muscle (Khassaf *et al.* 2001; McArdle *et al.* 2001) and similar mechanisms may therefore underlie the attenuation of the exercise-induced increase in HSP70 content following vitamin C supplementation. The vitamin C may have directly reduced the oxidants generated by muscle during exercise, maintained the redox environment of transcription factors or reduced oxidative damage to cellular proteins and again, the vitamin C-induced rise in HSP70 may have played a role.

Evaluation of the relative likelihood of these possibilities is difficult. We favour the possibility that either the vitamin C-induced increase in protective proteins (SOD, CAT or HSPs) reduced the stress on tissues sufficiently to negate the necessity for further adaptation, or that vitamin C acted to maintain the reducing environment of key molecules within the cell, for instance by maintenance of key thiol groups (McArdle *et al.* 2001) or preventing the oxidant-induced activation of specific transcription factors (Storz & Polla, 1996).

It is pertinent to consider whether the lack of an exercise-induced increase in HSP expression following vitamin C supplementation is beneficial or deleterious to the tissue.

There is increasing interest in the potential role of HSPs in maintenance of cell integrity and data from studies with cardiac and other tissues demonstrate that these proteins have significant cytoprotective effects against a variety of damaging processes (Marber *et al.* 1995). Our preliminary data from experimental animal studies indicate that these proteins can provide cytoprotective effects against contraction-induced damage to skeletal muscle (A. McArdle *et al.* 2002). The vitamin C-induced increase in baseline content of HSP70 may therefore be beneficial, but it is unclear whether suppression of the expression of these proteins following stress to skeletal muscle will be beneficial to skeletal muscle viability over the longer term. The relative validity of these two, apparently contradictory, conclusions will be evaluated by further studies of the importance of post-exercise HSP expression in the recovery and adaptation of skeletal muscle following contractile activity.

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